Effect of Ava II and NcoI Polymorphisms at the Low Density Lipoprotein Receptor Gene on Plasma Lipid Levels in a Group of Thai Subjects

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Abstract

The contribution of common genetic variations at the LDL receptor gene in determining interindividual differences in plasma lipid levels in the general population has been observed in several studies. In this study, we employed the PCR-RFLP method to investigate such an effect of the common Ava II (exon 13) and Nco I (exon 18) polymorphisms at the low density lipoprotein (LDL) receptor gene locus in 54 normolipidemic Thai subjects. The mean LDL-C level was slightly higher in the Ava II (+/+) genotype than the other Ava II genotypes. This difference was significant at the 5 per cent level although there were only three homozygotes with Ava II (+/+) genotype. The average effect of Ava II (+) allele was to increase LDL-C level by 6.75 mg/dl. A gene-dosage effect was not observed for this polymorphism. In addition, the subjects with Ava II (+/+) genotype also tended to have high serum total cholesterol and triglyceride and low HDL-C levels. Nco I polymorphism revealed no statistically significant effect on lipid levels in these subjects. However, the subjects with (+) allele tended to have high levels of LDL-C, serum total cholesterol and triglyceride.

Key word : LDL Receptor Gene, Ava II Polymorphism, Nco I Polymorphism

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The low density lipoprotein (LDL) receptor plays a crucial role in cholesterol homeostasis(1). A relatively high plasma level of LDL-cholesterol (LDL-C) has been recognized as a risk factor for coronary heart disease (CHD)(2). A mutation of the LDL receptor gene that disturbs the normal function

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of the receptor protein can cause familial hypercholesterolemia (FH)[3]. This genetic disorder is associated with elevated plasma LDL-C and premature CHD. However, FH accounts for only about 5 per cent of patients with CHD[2].

Common DNA polymorphisms in genes involved in lipid metabolism are potentially important genetic markers in affecting normal variation in the plasma lipid profile and thus determining susceptibility or resistance to CHD in the general population[2]. Early studies demonstrated that some common DNA polymorphisms at the LDL receptor locus affect plasma cholesterol levels in normal individuals. Such observations lead to the proposal that normal alleles at the LDL receptor locus may significantly contribute to the population variation in receptor activity and the lipid levels in serum and thus explains how normal genes can contribute to the level of risk factors for CHD[2,4,5]. In European populations, a common Pvu II (intron 15) polymorphism is associated with serum total cholesterol (TC) and LDL-C levels according to several[4,6-9] though not all studies[10,11]. In one study a gene-dosage effect on TC and LDL-C levels among Ava II (exon 13) and Nco I (exon 18) genotypes in normal populations was observed[2]. For these polymorphisms (considered separately), the lipid levels were low in the (-/-) genotype, intermediate in the (-/+) genotype, and high in the (+/+) genotype[2].

In this study, we investigated allele and genotype frequencies of the common Ava II (exon 13) and Nco I (exon 18) polymorphisms at the LDL receptor locus in 54 Thai subjects with normolipidemia. We also examined the role which these two common DNA polymorphisms may have in affecting plasma lipid profiles in these normolipidemic subjects. The Ava II polymorphism involves substitution of C for T in the third base of codon 632 (valine), which induces a recognition site for the enzyme Ava II. This mutation is synonymous so it does not change the amino acid. The Nco I polymorphic site is transcribed but not translated and therefore does not involve any amino acid change.

MATERIAL AND METHOD

Subjects

The subjects consisted of 54 apparently unrelated individuals (6 males, 48 females) of Thai ethnic background who attended hospital for regular clinical check ups at the Department of Preventive and Social Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University. None of these subjects were ever treated with any lipid-lowering drugs or other type of medication. Subjects with evidence of secondary hypercholesterolemia were excluded from this study. Those subjects whose serum TG levels exceeded 300 mg/dl were also excluded from this study.The mean (± SEM) for age and BMI were 47.55 (1.42) and 20.83 (0.31), respectively. The mean (± SEM) lipid levels, corrected for sex and age, were 210 (5.70), 128 (5.03), 57.90 (1.61) and 116.93 (9.28) for TC, LDL-C, HDL-C and triglyceride (TG), respectively. The age-and sex-specific means of TC and LDL-C levels used to define these normolipidemic subjects were as described previously[12].

Lipid analysis

Plasma cholesterol and triglyceride levels, in venous blood taken after 14 hours of fasting, were determined automatically using a Hitachi 917 Auto-analyzer. The concentration of plasma HDL –C was measured after precipitation of the LDL and VLDL fraction with dextran sulfate and magnesium chloride. Plasma LDL-C level was calculated by the formula of Friedewald *et al* as described previously[12].

Genotype analysis

Fasting EDTA blood samples were collected by venepuncture. DNA was extracted by the Guanidine-HCl method from UCLA tissue typing laboratory[13]. DNA samples were subjected to amplification by PCR in a Perkin-Elmer Cetus DNA thermal cycler (Gene Amp PCR System 2400, Perkin Elmer, USA). For Ava II RFLP analysis, The primers for PCR were A1 (5'-GACAAAGTATTTTGGA CAG-3') and A2 (5'-CTCTTGGCTGGGTGAGGT TG-3') [14]. For Nco I analysis the primers were N1 (5'-CAATCTTGTCGTTGATGG-3') and N2 (5'CAAACGATCCAGACTGGA GG-3')[14].

Amplification in a final volume of 25 ml contained approximately 25 ng of genomic DNA, 1xPCR buffer (supplied by the manufacturer as 10 x PCR), 5 pmol of each primer, 200 mM of each dNTP, and 0.5 unit of Taq DNA polymerase. dNTPs and Taq DNA polymerase were commercially supplied (Pharmacia Biotech, Sweden). Amplification of the region flanking the Ava II site was performed for 35 cycles at 95°C 1 min, 47°C 1 min and 72°C 2 min. Amplification of a region in exon 18 for Nco I restriction analysis by N1 and N2 primers was performed for 35 cycles at 95°C 1 min, 55°C 1 min

and 72°C 2 min. The amplified products were relevantly digested with Ava II (Biolabs,UK) and Nco I (Biolabs,UK) and the resulting fragments were separated by agarose gel electrophoresis.

Statistical analysis

Allele and genotype frequencies for each polymorphic site were estimated by gene counting. The frequency of the genotype groups compared to that expected for Hardy-Weinberg proportions was analysed by a X^2-test for both Ava II and Nco I polymorphisms. Differences in mean lipid levels between genotypes of Ava II and Nco I polymorphisms were evaluated by nonparametric (Kruskal-Wallis) and parametric (Unpaired *t*-test) tests. Statistical significance was taken at a level of $p<0.05$. The average effect of LDL receptor alleles for Ava II polymorphism was estimated according to the method of Boerwinkle *et al.* as described previously[4,15].

RESULTS

The PCR products of exon 13 amplified with A1 and A2 were 142 bp in size. Digestion of these PCR products with Ava II revealed two fragments of 111 and 31 bp, indicating the presence of a restriction site (+ allele). The products amplified with N1 and N2 primers were 859 bp in size. The digestion of these fragments with Nco I yielded two fragments of 480 and 379 bp for the allele containing the Nco I site (+ allele). The (+) allele frequencies of Ava II and Nco I were 0.24 and 0.61, respectively. The genotype distribution of Ava II polymorphism was 57.41 per cent, 37.04 per cent and 5.56 per cent for the (-/-), (-/+) and (+/+) genotypes, respectively. The genotype distribution of Nco I polymorphism was 12.96 per cent, 51.85 per cent and 35.19 per cent for the (-/-), (-/+) and (+/+) genotypes, respectively. From the X^2-test, the observed values of genotype distribution of both polymorphisms were not significantly different from expected values ($p>0.05$). Therefore, both Ava II and Nco I genotypes were in Hardy-Weinberg equilibrium. The results were shown in Table 1.

The effects of the genotypes on lipid levels were examined for each restriction site separately. Comparison of the mean lipid levels among groups of individuals with different genotypes of both

Table 1. Genotype frequencies of the Ava II (exon 13) and Nco I (exon 18) polymorphisms at the LDL receptor gene in Thai subjects (n = 54; total alleles = 108).

	Ava II genotypes [a]			Nco I genotypes [b]		
	-/-	-/+	+/+	-/-	+/+	+/+
Observed	31	20	3	7	28	19
Expected	30.73	19.98	3.24	8.1	25.38	19.98

a. $X^2 = 0.01$ (not significant ($p>0.05$)) b. $X^2 = 0.45$ (not significant ($p>0.05$))

Table 2. Plasma lipid traits [mean ± (SEM)] in subjects with different Ava II genotypes.

Trait	Ava II genotypes			p
	-/- (n=31)	-/+ (n=20)	+/+ (n=3)	
LDL-C*	131.45 (6.05)	118.48 (8.84)	173.67 (4.84)	<0.05
Chol	213.07 (6.89)	200.05 (10.29)	253.00 (12.06)	0.13
HDL-C	58.72 (2.19)	57.71 (50.63)	50 (2.84)	0.39
TG	111.48 (12.64)	121.80 (15.54)	140.67 (21.88)	0.43

* statistical significance was observed for both nonparamatric (Kruskal-Wallis) and paramatric (Unpaired-T test) analyses

Table 3. Plasma lipid traits [mean ± (SEM)] in subjects with different Nco I genotypes.

Trait	Nco I genotypes			p
	-/- (n=31)	-/+ (n=20)	+/+ (n=3)	
LDL-C	113.17 (13.79)	130.97 (7.53)	131.90 (7.53)	0.40
Chol	194.29 (17.50)	212.71 (8.08)	213.58 (9.15)	0.44
HDL-C	59.29 (2.93)	59.26 (2.07)	55.37 (3.23)	0.45
TG	109.57 (34.12)	109.00 (10.74)	131.32 (17.44)	0.55

polymorphisms were performed by nonparametric (Kruskal-Wallis) and parametric (Unpaired *t*-test) tests. Table 2 and 3 show means of TC, LDL-C, HDL-C and TG levels among three genotypes of Ava II and Nco I polymorphisms, respectively. The mean LDL-C level was slightly higher in the Ava II(+/+) genotype than the other Ava II genotypes. Although, only three homozygotes with the Ava II (+/+) genotype were observed, this difference was statistically significant at the 5 per cent level ($p<0.05$) for both Kruskal-Wallis and Unpaired *t*-test analyses. Such significant differences in mean levels of TC, TG and HDL-C were not observed among Ava II genotypes. However, the subjects with Ava II (+/+) genotype tended to have higher TC and TG and lower HDL-C levels than the other two genotypes.

The mean (± SEM) LDL-C level (corrected for age and sex) in this sample was 128.00 (± 5.03) mg/dL. The mean effect of Ava II (+) allele was to increase LDL-C level by 6.75 mg/dL. However, the effect was not gene-dosage dependent because the mean LDL-C level was significantly higher in Ava II (-/-) genotype than the Ava II (-/+) genotype ($p<0.05$). In conclusion, among the three Ava II genotypes the mean LDL-C level was lowest in the Ava II (-/+) genotype.

For Nco I polymorphism, no significant variability among genotypes was observed for the lipid traits. However, there were trends for subjects with (+) allele (both -/+ and +/+ genotypes) to possess slightly higher mean levels of LDL-C, TC and TG.

DISCUSSION

We investigated the distribution of two common DNA polymorphisms, Ava II (exon 13) and Nco I (exon 18), in the LDL receptor gene and evaluated their effect on plasma lipid levels in a group of Thai subjects with normolipidemia.

From this study, the frequency of the (+) allele for Ava II and Nco I polymorphisms at the LDL receptor was remarkably different from those reported in Caucasian populations[2,14,16,17] and also from a Japanese population[18]. This may be due to differences in ethnic background and geographic isolation. However, an exception was noted. The Nco I (+) allele frequency in our subjects (0.61) was close to that found in Germans (0.60).

The results from our study provide evidence that common genetic variation at the LDL receptor gene potentially affect lipid levels in normolipidemic individuals. LDL-C level appears to be a slightly better marker for the gene than the total plasma cholesterol levels[19]. Besides, a relatively high plasma level of LDL-C has been found to be a risk factor for CHD[2]. Our data revealed that the mean level of LDL-C was significantly higher in subjects with the Ava II (+/+) genotype than the other two genotypes. Furthermore, the subjects with the Ava II (+/+) genotype also tended to have high levels of TC and TG and low levels of HDL-C. The average effect of the Ava II (+) allele was to increase LDL-C level by 5.27 per cent (6.75 mg/dl). However, the effect was not gene-dosage dependent as previously reported in another study[2] because the mean LDL-C level was significantly higher in subjects with Ava II (-/-) genotype than the Ava II (-/+) genotype. The kind of association observed in this study might be due to chance alone because only three subjects with Ava II (+/+) genotype were observed. Otherwise, interactions among different genes and/ or polymorphisms of these genes involved in lipid metabolism may account for this non-gene-dosage effect.

For Nco I polymorphism, the subjects with (+) allele (both -/+ and +/+ genotypes) tended to have high levels of TC and LDL-C. The subjects with Nco I (+/+) genotype also tended to have a higher level of TG than the other two genotypes. However, these effects did not reach statistical significance.

These effects did not reach statistical significance. It is well accepted that lipid levels are determined by both genetic and environmental factors and, for the genetic part, more than one gene is involved. By definition, lipid levels are thus continuous or quantitative traits, displaying a continuous distribution of phenotypes[20]. Such continuous traits with small difference of phenotypes (i.e., concentration of lipid levels in this study) seem to lead to a non-statistically significant association between lipid levels and genotypes. Therefore, the simple relationship between genotype and phenotype that exists in discontinuous traits are not clearly or significantly observed in this study and thus only trends were observed. The exception was the statistically significant effect of Ava II (+/+) genotype on the LDL-C level. However, the number of the subjects with this genotype seemed to be too small (only 3 individuals) for a strong or more meaningful conclusion. Some evidence from the literature suggests that there may be common allelic variations in the LDL receptor gene that have small (less than 1 standard deviation from the mean) effects on interindividual lipid variation[5]. Haviland *et al.* previously reported the detection of such LDL allele effects of less than half of a standard deviation from the mean [5]. Therefore, the number of subjects for this kind of study should be larger (in this study, n=54) in order to obtain a statistically significant effect of normal LDL receptor allele.

In conclusion, the common genetic variations, Ava II (exon 13) and Nco I (exon 18), in normal alleles of LDL receptor gene potentially exert a small average effect on lipid levels. The mechanism underlying such an effect is not known. These polymorphisms may be functional DNA sequence variations responsible for variation in traits which are continuously distributed among individuals in population, i.e., in the similar way as apo E polymorphisms affect population variance in LDL-C levels[21]. However, another possible interpretation is that these polymorphisms may be in linkage disequilibrium with functionally important parts of the LDL receptor locus.

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การศึกษาผลของ polymorphism Ava II และ Nco I ในยีน LDL receptor ต่อระดับไขมันในเลือดของคนไทย

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จากการศึกษาเกี่ยวกับระดับไขมันของคนปกติในหลายเชื้อชาติพบว่า polymorphism ของยีน LDL receptor มีผลทำให้เกิดความแตกต่างของระดับไขมันในเลือดของคนปกติเหล่านั้น การศึกษานี้ได้ใช้เทคนิค PCR-RFLP ดูผลดังกล่าวของ polymorphism 2 ตำแหน่ง ในยีน LDL receptor (คือ Ava II ใน exon 13 และ NcoI ใน exon 18) ในคนไทยที่มีระดับไขมันในเลือดปกติจำนวน 54 คน พบว่าคนที่มี Ava II (+/+) genotype จะมีระดับคลอเลสเตอรอลรวม (TC), แอลดีแอลคลอเลสเตอรอล (LDL-C), ไตรกลีย์เซอไรด์ (TG) สูง และมีระดับเอชดีแอลคลอเลสเตอรอล (HDL-C) ต่ำกว่าคนที่มี genotype เป็น Ava II (-/-) และ Ava II (-/+) อย่างไรก็ตามเฉพาะระดับ LDL-C เท่านั้นที่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติที่ระดับความเชื่อมั่น 5% และจากการคำนวณพบว่า Ava II (+) allele อาจมีผลทำให้ระดับ LDL-C เพิ่มขึ้นประมาณ 6.75 mg/dL แต่การเปลี่ยนแปลงของระดับ LDL-C นี้ไม่ได้มีลักษณะเป็นแบบ gene-dosage effect สำหรับ Nco I polymorphism นั้นพบว่าคนที่มี Nco I (+) allele มีระดับ TC, LDL-C และ TG ค่อนข้างสูงกว่าคนที่มี Nco I (-) allele แต่ผลดังกล่าวนี้ไม่มีนัยสำคัญทางสถิติ

คำสำคัญ : ยีน LDL receptor, Ava II polymorphism, Nco I polymorphism

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